

## G PROTEIN-INDEPENDENT SIGNALING OF AT<sub>1</sub>-R

Angiotensin II (AngII) is the main regulator of the renin-angiotensin system and participates not only in physiological but also in pathological mechanisms. The type I angiotensin receptor (AT<sub>1</sub>-receptor) is a typical G protein-coupled receptor and largely acts via G<sub>q</sub> activation upon agonist binding and these G protein-mediated „classical” signaling mechanisms are responsible for the most majority of AngII evoked cellular responses. Recently, new type of AT<sub>1</sub>-receptor mediated signaling mechanisms are discovered where the Ang II-induced signal transduction steps are not dependent on G protein coupling („G protein-independent”). By the help of the NNF-OTKA grant, we investigated the G protein-independent signaling of AT<sub>1</sub>-receptor in living cells using wide range of molecular and cellular biology methods. All the experiments were performed in the Department of Physiology, Semmelweis University.

### Publication:

András Balla, László Sándor Erdélyi, Eszter Soltész-Katona, Tamas Balla, Péter Várnai, László Hunyady: ***Demonstration of Angiotensin II-Induced Ras Activation in the Trans-Golgi Network and the Endoplasmic Reticulum Using BRET-Based Biosensors***, Journal of Biological Chemistry, 2011, 286(7):5319-27

### Manuscript:

András Balla, Eszter Soltész-Katona, Dániel Tóth, Gyöngyi Szakadati, László Sándor Erdélyi, Péter Várnai, László Hunyady: ***Mapping of molecular dynamics of type I angiotensin receptor upon stimulus detected by bioluminescence resonance energy transfer-based sensors***

### Conference abstracts, and poster presentations:

Andras Balla, Peter Varnai, Laszlo Hunyady: ***Molecular dynamics of type I angiotensin receptor upon stimulus***, Gordon Research Conference on Angiotensin, 2010 (www.grc.org), 2010

Andras Balla, Eszter Soltész-Katona, Laszlo Sandor Erdelyi, Peter Varnai, Laszlo Hunyady: ***Molecular dynamics of type I angiotensin receptor upon stimulus***, ASCB 50th Annual Meeting, 2010, 2010

András Balla, László Sándor Erdélyi, Eszter Soltész-Katona, Tamas Balla, Péter Várnai, László Hunyady: ***Detection of Angiotensin II-Induced Ras Activation in the Trans-Golgi Network and the Endoplasmic Reticulum Using BRET-Based Biosensors***, Gordon Research Conference on Molecular Pharmacology, 2011 (www.grc.org), 2011

András Balla, Eszter Soltész-Katona, Dániel Tóth, László Sándor Erdélyi, Péter Várnai, László Hunyady: ***Molecular dynamics of type I angiotensin receptor upon stimulus***, Semmelweis Symposium: Cellular Signaling in Physiology and Pathology, 2010, Budapest

Oral presentations:

Balla András, Erdélyi László, Soltész-Katona Eszter, Várnai Péter, Hunyady László: ***Intracelluláris kis G-fehérje aktiválódás vizsgálata az angiotenzin hatásmechanizmusában***, Magyar Biokémiai Egyesület 2010. évi Vándorgyűlése; 2010. augusztus 25-28., 2010

/planned/ Balla András: ***Agonisták hatása a receptorok plazmamembrán mikrodomén lokalizációjára***, Magyar Biokémiai Egyesület Gyógyszerbiokémiai Szakosztály munkaértekezlete, Balatonőszöd; 2011.

Andras Balla has participated in scientific meetings by the support of NNF-OTKA grant:

- Gordon Research Conference on Angiotensin, 2010 (www.grc.org), Ventura, USA
- ASCB 50th Annual Meeting, 2010, Philadelphia, USA
- Magyar Biokémiai Egyesület Vándorgyűlése; 2010, Budapest
- Semmelweis Symposium 2010, Budapest

Results:

***Demonstration of Angiotensin II-Induced Ras Activation in the Trans-Golgi Network and the Endoplasmic Reticulum Using BRET-Based Biosensors***

Since this work is already published in the Journal of Biological Chemistry, only a summary (without figures) is provided:

The small G-proteins such as Ras, Rho and Rac are central players of many signal transduction pathways and regulate a wide variety of cell functions. Recently discovered that extracellular stimuli of G-protein-coupled receptors, including AT<sub>1</sub>-receptor can lead to activation of various small G-proteins. It is generally accepted that the signal generation of GPCRs occurs at the plasma membrane. Demonstration of the presence of signaling molecules in intracellular compartments raises the question, if signal generation can occur during receptor internalization. In order to investigate small G-protein activation upon agonist stimulus of AT<sub>1</sub>-receptor we have developed several intra- and intermolecular probes in bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET) measurements and confocal microscopy in living cell experiments (Balla *et al*, JBC, 2011) by the means of NNF-OTKA grant. We have also targeted our probes to various cellular locations thus we are able to monitor small G-protein activation in different plasma membrane and intracellular compartments. BRET probes report energy transfer from an energy donor (*Renilla* luciferase) to YFP when Ras become loaded with GTP and interacts with its effector during Ras activation. In our studies we used BRET measurements, since BRET has several advantages compared to FRET microscopy. For example, BRET is a more quantitative method, because it can be performed on large population of cells. BRET is also more sensitive compared to FRET, since the excitation light causes high background in FRET measurements. Our BRET probes were targeted to plasma membrane microdomains and intracellular organelles, including endoplasmic reticulum (ER), and trans-Golgi network (TGN), using specific targeting sequences to assess the effect of hormonal stimulation on Ras activity in these compartments.

AngII induced a transient Ras activation in the plasma membrane, which was much smaller compared to the EGF-induced response. This finding is consistent with the fact that EGF is a more effective Ras activator than AngII, however, it is possible that the incomplete transfection efficiency of the angiotensin receptor containing plasmid also contributed to this finding. Kinetically, EGF receptor activation also

produced more prolonged Ras activation compared to the AngII-induced response. We were able to demonstrate that AngII stimulation not only increases Ras activity in the plasma membrane, but can also activate it in intracellular compartments, such as the TGN and the ER. We have also detected AngII-induced rapid and transient Ras activation in the TGN. AngII was a relatively efficient activator of Ras in the TGN, since the amplitude of the AngII and EGF responses in the TGN were comparable, whereas EGF caused a much larger response in the plasma membrane. AngII caused a more prolonged activation of Ras in the TGN in cells expressing the internalization-deficient AT<sub>1A</sub>-R Δ319, which is consistent with the impaired desensitization of this receptor, and argue against the role of receptor endocytosis in AngII-induced Ras activation in the TGN. The Ras activation in the ER is surprising in many respects. It is remarkable that only AngII stimulus activated Ras, but EGF receptor stimulation was not able to produce this effect. This result provides evidence that the effect of AngII in the ER is independent of EGF receptor transactivation. Similar to the TGN, AngII caused a more prolonged activation of ER-targeted Ras in cells expressing the internalization-deficient AT<sub>1A</sub>-R Δ319, suggesting that the effect of AngII on Ras activation is also internalization independent in this compartment.

The mechanisms of AngII-induced Ras activation in the plasma membrane is cell type dependent. In vascular smooth muscle cells, and many other cell types transactivation of EGF receptors plays a major role in the mitogen effects of AngII. In our study AG1478, an EGF receptor kinase inhibitor, eliminated all EGF-induced responses, but had no effect on AngII-induced Ras activation in the Golgi and the ER, similar to that in the plasma membrane, in HEK293 cells. This finding is consistent with earlier studies, which demonstrated that AngII-induced ERK activation and mitogen signaling is independent of EGF receptor transactivation in these cells. Considering its rapid timing, it is likely that the effect of AngII on Ras activation in the TGN and the ER is mediated by soluble messengers, similar to the previously reported similar effects of growth factors and LPA. In cells expressing the internalization-deficient AT<sub>1A</sub>-R Δ319 mutant AngII caused more prolonged Ras activation both in the plasma membrane and in endomembranes, which is probably caused by the impaired desensitization of this mutant receptor. These data are also consistent with our conclusion that AngII-induced Ras activation in the Golgi and the

ER is G protein mediated, and does not require receptor internalization or  $\beta$ -arrestin-mediated signaling.

We did not detect significant AngII-induced Ras activation in cells expressing DRY/AAY mutant of AT<sub>1</sub>-R suggesting that the response, both in the plasma membrane and in endomembranes, is predominantly G protein mediated. We also have investigated the Ras activation by utilization of a biased AT<sub>1</sub>-receptor agonist, [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII, which activates only G protein-independent pathways. In accord with the findings using DRY/AAY mutant of AT<sub>1</sub>-R, the [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII stimulus did not yield significant Ras activation in HEK293 cells. We tried to measure Ras activation upon stimulus of endogenous AT<sub>1</sub>-receptor. We isolated smooth muscle cells from rats, but we were not able to detect significant Ras activation upon AngII treatment. It is likely, that the isolated cells lost their AT<sub>1</sub>-receptors during the cell grow since large number of cells is required for BRET experiments.

***Mapping of molecular dynamics of type I angiotensin receptor upon stimulus detected by bioluminescence resonance energy transfer-based sensors***

Since this work is not yet published in peer-reviewed journal, a detailed report is provided: In the these sets of experiments we investigated the dynamics of AT<sub>1</sub>-receptor movement, such as internalization or lateral movement between plasma membrane compartments upon hormone stimulus in BRET measurements. In order to analyze the G-protein independent components of AT<sub>1</sub>-receptor trafficking, we compared the data of AT<sub>1</sub>-receptor upon AngII or [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII stimuli. We also investigated the effect of introducing DRY/AAY mutation into the receptor, hence we could measure the G-protein independent mechanisms in the molecular dynamics of AT<sub>1</sub>-receptor. Since, this work is not published yet, I would like to show our results in details:

The concept that the plasma membrane is not a uniform structure, but rather a mixture of microdomains has provided the possibility of compartmentalized signaling. The membrane rafts are specific microdomains of the plasma membrane and they differ in their compositions from the rest of the plasma membrane. These cholesterol- and sphingolipid-rich plasma membrane microdomains play important roles in compartmentalization of cellular functions. The membrane rafts are frequently referred as lipid rafts whereas the rest of the plasma membrane can called as non-lipid

rafts or disordered membrane. Formation of rafts requires cholesterol and the cholesterol depletion by  $\beta$ -methyl-cyclodextrin (M $\beta$ CD) treatment is widely used for lipid raft disruption. The membrane microdomains are located in both leaflets of the plasma membrane. It was also demonstrated by FRET approach that a lipid anchor on a fluorescent protein is sufficient to sequester to different microdomains within the plasma membrane. Zaccharias *et al.* constructed YFP fused with short peptides containing consensus sequences for acylation such as myristoylation and palmitoylation (MyrPalm-YFP, MP-YFP).

Although, the roles of membrane rafts are hot topics in the literature but their existence remain challenged. It is well established that some structural motifs of plasma membrane proteins are responsible for targeting into a membrane microdomains, and several membrane markers were recently developed to investigate membrane microdomains. Since the concept of membrane rafts are based on biochemical experiments utilizing various detergent extraction methods we decided to use other approach to investigate the relation of AT<sub>1</sub>-R to membrane microdomains in living cells. Earlier studies have demonstrated that AngII stimulation of AT<sub>1</sub>-R promotes its association and trafficking into caveolin-enriched/lipid rafts in vascular smooth muscle cells. Instead of focusing the characterization of the biophysical and biochemical nature of the plasma membrane microdomains during AT<sub>1</sub>-receptor action we followed the distribution of the AT<sub>1</sub>-receptor during its action. FRET and BRET based methods are widely used in GPCR research studies such as investigation of receptor oligomerization and lateral distribution of GPCRs. The main advantage of these methods that the measurements can be performed in living cells. We used several yellow fluorescent protein (YFP)-labeled fusion constructs (i.e. raft-, or non-raft plasma membrane markers) to analyze the dynamics of AT<sub>1</sub>-R movement, such as internalization or lateral movement between plasma membrane compartments upon stimulus in BRET measurements. The BRET probes are report energy transfer from an energy donor (*Renilla* luciferase) to an acceptor (YFP). When the luciferase is close to the YFP we can detect BRET signal and upon diverge from each other (or if the orientation is changed) the BRET ratio signal drops. In contrast to that, when luciferase and YFP come up closer to each other an elevated BRET ratio signal can be detected.

We constructed numerous constructs by the help of NNF-OTKA grants. We used the eYFP-C1 or eYFP-N1 plasmid backbone for the construction of YFP labeled constructs. AT<sub>1</sub>R-Rluc was constructed by replacing the eYFP coding region in AT<sub>1</sub>R-YFP with *Renilla* luciferase. The luciferase tagged 5-hydroxytryptamine receptor-2C receptor (5HT-2CR) was constructed by subcloning the receptor cDNA into the codon humanized *Renilla* luciferase pRluc-N1 vector. The luciferase tagged epidermal growth factor receptor (EGFR) was constructed by replacing the eYFP coding region in EGFR-YFP with *Renilla* luciferase. The MP (MyrPalm) and PP (PalmPalm) targeting was the N-terminal MGCIKSKRKDNLNDDE amino acid sequence from Lyn kinase, and PP was the N-terminal MLCCMRRTKQ amino acid sequence from Gap43. In order to target the eYFP to the disordered plasma membrane microdomain, we have fused C-terminally the CAAX motif from K-Ras small G protein (tK stands for the K-Ras CAAX targeting motif, tail K-Ras), KMSKDGKKKKKKSKTKCVIM consists of the membrane targeting CAAX motif and hypervariable regions of K-Ras. The DRY/AAY mutation of the AT<sub>1A</sub>-R (the highly conserved D<sup>125</sup>R<sup>126</sup>Y<sup>127</sup> was mutated to A<sup>125</sup>A<sup>126</sup>Y<sup>127</sup>) and the TSTS/A mutant of the AT<sub>1A</sub>-R (the T<sup>332</sup>S<sup>335</sup>T<sup>336</sup>S<sup>338</sup> were substituted with alanine) were described earlier. The experiments were performed on HEK293 cell line. The cells were cultured in DMEM with Pen/Strep and 10% heat-inactivated fetal bovine serum in 5% CO<sub>2</sub> at 37 °C. The cells were cultured in plastic dishes and were trypsinized prior to transfection and were transiently transfected by using Lipofectamine 2000 and plated on poly-lysine pretreated white 96-well plates in 1x10<sup>5</sup> cells/well density for BRET measurements. The DNA amounts were 0.25 µg Rluc containing construct/well and 0.0625 µg YFP containing construct/well; the amount of Lipofectamine 2000 was 0.5 µl/well. The BRET measurements were performed after 24 h of the transfection on white 96-well plates. The medium of the cells were changed prior to measurements to a modified Krebs-Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.7 mM MgSO<sub>4</sub>, 10 mM glucose, and Na-HEPES 10 mM, pH 7.4; and the BRET measurements were performed at 37 °C. The BRET measurements were started after addition of the cell permeable substrate, coelenterazine at a final concentration of 5 µM, and the counts were recorded by using Berthold Mithras LB 940 multilabel reader that allows for the detection of signals using filters at 485 and 530 nm wavelengths, the detection time was 0.25-0.5 sec. The BRET ratios were calculated as 530 nm/485 nm ratio. Measurements were done in triplicate. The BRET records are

average of at least 3 independent experiments. BRET ratios were baseline-corrected to the vehicle curve using GraphPad Prism software. The approximate BRET ratio using the cytosolic eYFP (unbiased) and AT<sub>1</sub>R-Rluc pair is ~0.85. The localization and distribution of the targeted probes was analyzed using Zeiss LSM 510 confocal laser-scanning microscope in living cells plated on poly-lysine pretreated glass coverslips (3x10<sup>5</sup> cells/35-mm dish).

*BRET assay for detection of dynamics of AT<sub>1</sub>-R:* To detect the movement of AT<sub>1</sub>-R upon stimulus, our strategy was to use *Renilla* luciferase labeled AT<sub>1</sub>-R and we followed the BRET ratio between the AT<sub>1</sub>-R and a YFP labeled protein counterpart. When the HEK293 cells co-expressing wild type AT<sub>1</sub>-R-luciferase and arrestin-YFP were exposed to 100 nM AngII the BRET ratio (Fig. 1A, filled rectangles) elevated between the arrestin-YFP and wild-type AT<sub>1</sub>-R-luciferase showing that the  $\beta$ -arrestin binds to the activated AT<sub>1</sub>-R. The internalized AT<sub>1</sub>-R then appears in the endocytotic route, which could be detected with the energy transfer between the Rab5-YFP (as early endosome marker) and the wild-type AT<sub>1</sub>-R-luciferase (Fig. 1B, filled rectangles). When the plasma membrane marker MP-YFP was used the BRET ratio dropped upon stimulus of the wild type AT<sub>1</sub>-R-luciferase (Fig. 1C, filled rectangles), but in contrast to that, when KR-YFP (also plasma membrane located similar to MP-YFP) was used we measured a significant BRET signal elevation prior to drop in BRET ratio (Fig. 1D, filled rectangles). We think that the observed changes in the BRET ratio upon stimulus of AT<sub>1</sub>-R-luciferase using either MP-YFP or KR-YFP are not consequence of the high protein overexpression. The gradual reduction of the DNA amounts used for the transfection, resulting very low counts in the BRET measurements, did not alter the shapes and extents of the BRET ratio changes (data not shown). Since the cytosolic YFP and AT<sub>1</sub>-R are not in the same compartment, the stimulus of the AT<sub>1</sub>-R-luciferase by 100 nM AngII did not change the BRET ratio (Fig. 1E, filled rectangles).

*Molecular dynamics of mutant AT<sub>1</sub> receptors:* The dissimilar BRET curves of MP-YFP or KR-YFP raised the possibility that we could monitor the movement of AT<sub>1</sub>-R between different plasma membrane compartments in living cells immediately in response to agonist stimulus. We further investigated whether the dynamics of the receptor is altered using mutant AT<sub>1</sub>-Rs (Fig. 1 open rectangles, dotted lines: DRY/AAY AT<sub>1</sub>-R; open circles: TSTS/A AT<sub>1</sub>-R). It was revealed that the dynamics of DRY/AAY AT<sub>1</sub>-R is dramatically changed compared to wild type AT<sub>1</sub>-R. When



the DRY/AAY AT<sub>1</sub>-R-luciferase was exposed to 100 nM AngII, the  $\beta$ -arrestin binding capability of the Ang II-bound DRY/AAY mutant receptor is slightly decreased (Fig. 1A, open rectangles, dotted lines). The AngII stimulated DRY/AAY mutant receptor was also able to translocate to Rab5 endocytic compartments (Fig. 1B, open rectangles, dotted lines). Moreover, the BRET ratio drop with MP-YFP is more immediate using DRY/AAY AT<sub>1</sub>-R-luciferase (Fig. 1C, open rectangles, dotted lines) compared to wild type AT<sub>1</sub>-R-luciferase was used the BRET ratio (Fig. 1C, filled rectangles). Using the DRY/AAY AT<sub>1</sub>-R-luciferase and the KR-YFP the BRET ratio (Fig. 1D, open rectangles, dotted lines) decreased without the initial elevation compared to the results using the wild type AT<sub>1</sub> receptor after the stimulus of HEK293 cells with 100 nM AngII (Fig. 1D, filled rectangles). We also determined the molecular dynamics of another AT<sub>1</sub>-R mutant, TSTS/A mutant which is not able to bind  $\beta$ -arrestin and its internalization is significantly reduced in response to AngII stimulus (Fig. 1A and 1B, open circles). Since the internalization of the TSTS/A AT<sub>1</sub>-R is reduced compared to wild type AT<sub>1</sub>R, the slow decrease in BRET ratios are reduced (Fig. 1C and 1D, open circles). We think that the slow decrease component of the BRET signal is the consequence of the internalization of the AT<sub>1</sub>-R, which results in diminishing AT<sub>1</sub>-R from the plasma membrane. This assumption is confirmed using AT<sub>1</sub>-R-luciferase and PLC $\delta$ 1-PH-YFP as BRET pairs.

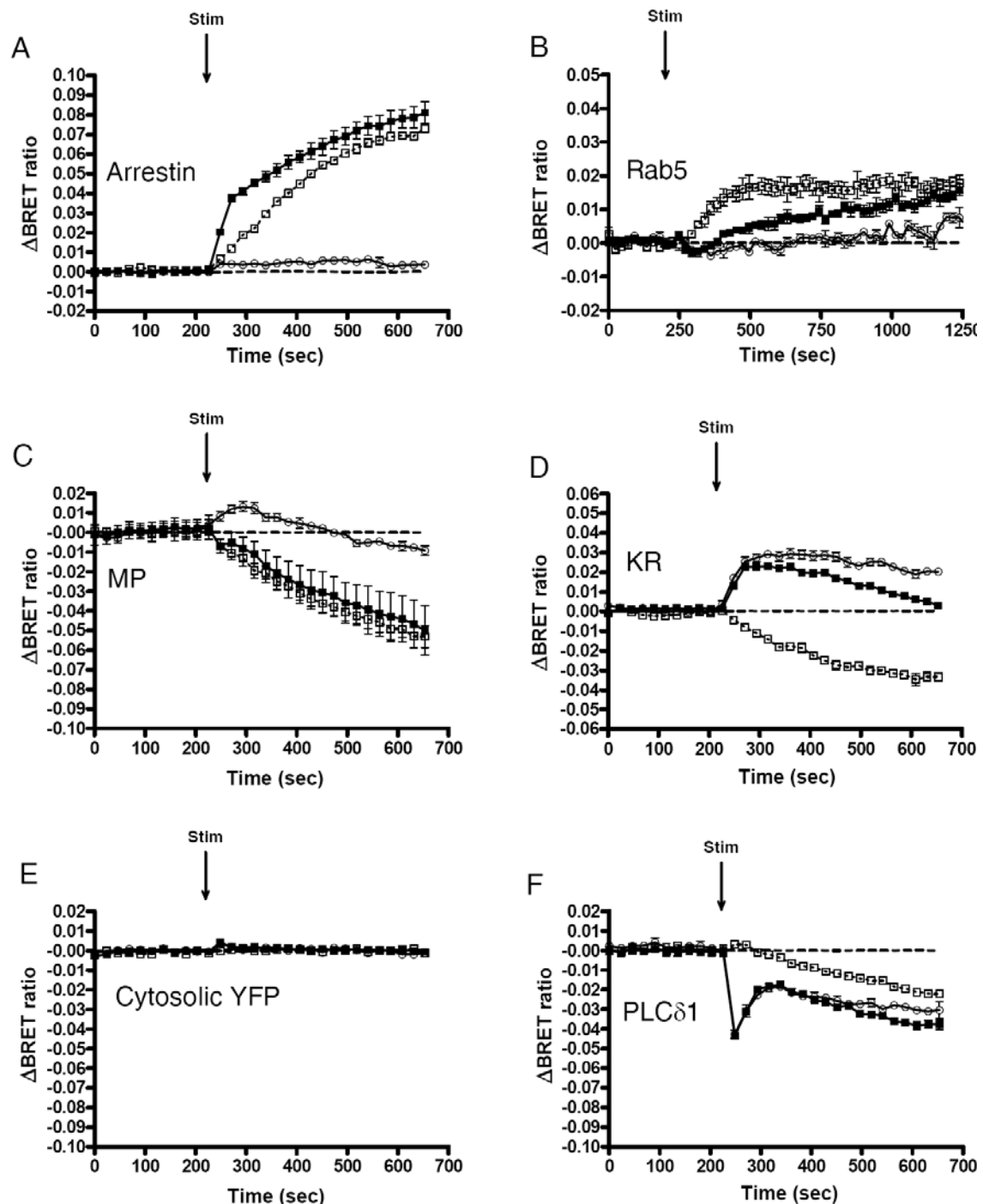


Figure 1. BRET assay between AT<sub>1</sub>-R and different proteins upon AngII stimulus in HEK293 cells. HEK293 cells were transfected with the plasmids of the indicated AT<sub>1</sub>-R-luciferase (filled rectangles: wild type; open rectangles, dotted lines: DRY/AAV mutant; open circles: TSTS/A mutant) and with the indicated YFP fused proteins, and after 24 hours the cells were exposed to 100 nM AngII or vehicle (dashed line) at the indicated time points. The BRET records are average of 3 independent experiments. Mean values  $\pm$  SEM are shown (n = 3).

When the HEK293 cells co-expressing wild type AT<sub>1</sub>-R-luciferase and PLC $\delta$ 1-PH-YFP were exposed to 100 nM AngII the BRET ratio dropped very quickly then the

ratio started to increase (Fig. 1F, filled rectangles). This initial drop shows that the AT<sub>1</sub>R-luciferase and the PLC $\delta$ 1-PH-YFP diverged from each other then they started to approach other. The PH domain of PLC $\delta$ 1 is a marker for PtdIns(4,5) $P_2$  in the plasma membrane. The initial drop in the BRET signal reflects that the agonist stimulus of AT<sub>1</sub>-R cause PtdIns(4,5) $P_2$  breakdown via G<sub>q</sub>, which results the release of PLC $\delta$ 1-PH-YFP from the plasma membrane. After short period of time the ratio begins to elevate since the PtdIns(4,5) $P_2$  resynthesis occurs very rapidly and the PLC $\delta$ 1-PH-YFP rebinds to the plasma membrane. This elevation in the BRET signal is followed by a slow decrease, which suggests that the AT<sub>1</sub>-R-luciferase and the PLC $\delta$ 1-PH-YFP starts to diverge from each other again because of the receptor internalization. When the HEK293 cells co-expressing DRY/AAY AT<sub>1</sub>-R-luciferase and PLC $\delta$ 1-PH-YFP was exposed to 100 nM AngII the BRET ratio decreased continually without a peak in the drop (Fig. 1F, open rectangles, dotted line). This reflects the fact that the stimulus of the DRY/AAY AT<sub>1</sub>-R does not result PtdIns(4,5) $P_2$  breakdown (release of PLC $\delta$ 1-PH-YFP from the plasma membrane, Fig. 1F) but the DRY/AAY AT<sub>1</sub>-R internalizes upon stimulus (Fig. 1B, open rectangles, dotted lines).

*Distribution of plasma membrane makers upon agonist stimulus:* We examined the distribution of MP-YFP, PLC $\delta$ 1-PH-YFP, and KR-YFP in HEK293 cells in response to AngII stimulus. As shown in the Fig. 2, the AngII stimulus did not alter noticeably the distribution and amount of MP-YFP or KR-YFP in the plasma membrane. In contrast to that the PLC $\delta$ 1-PH-YFP temporarily is translocated to the cytoplasm and then returns to the plasma membrane reflecting the PtdIns(4,5) $P_2$  level in the plasma membrane. Taken together, it seems that the changes in the BRET ratio using MP-YFP or KR-YFP along with AT<sub>1</sub>-R-luciferase reflect the alterations in the AT<sub>1</sub>-R distribution and not the changes in the using MP-YFP or KR-YFP distribution in the plasma membrane.

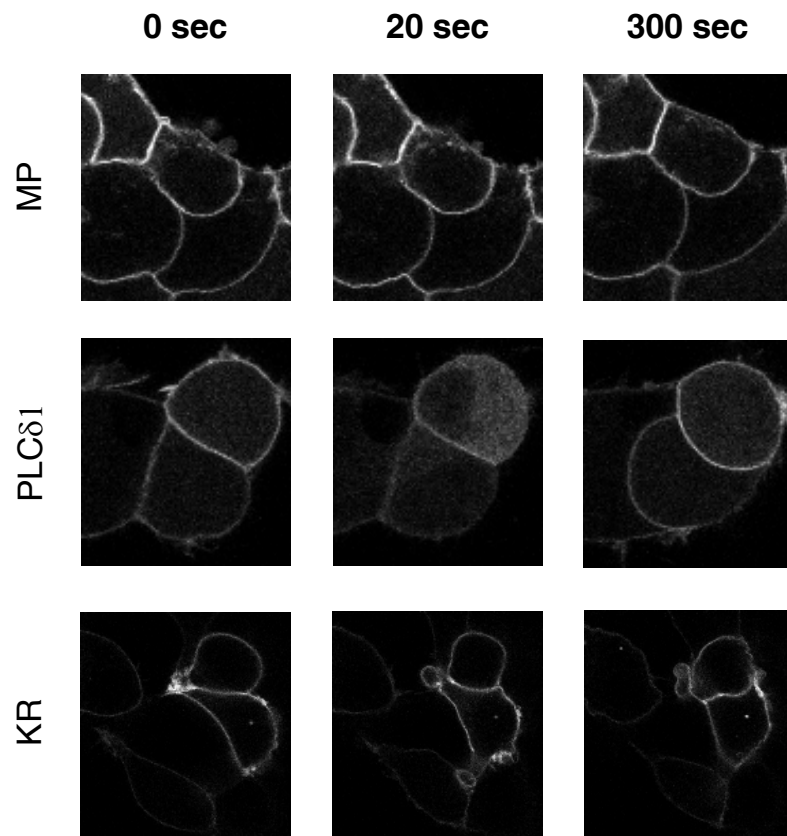


Figure 2. Effects of 100 nM AngII stimulus on the distribution of MP-YFP, PLC $\delta$ 1-PH-YFP, and KR-YFP in HEK293 cells. The cells were transfected with the AT<sub>1</sub>-R-luciferase and with the indicated YFP fused proteins. After 24 hours the cells were exposed to 100 nM AngII. The probes were visualized by laser scanning confocal microscopy (Zeiss LSM510). The representative confocal micrographs show the localization and cellular distribution of the indicated probes before (0 sec) and 20 sec or 300 sec after the AngII treatment. The YFP fluorescence was detected by Zeiss LSM 510 confocal microscope. Bars: 10  $\mu$ m.

*Effect of cholesterol depletion on the AT<sub>1</sub>-R motion upon agonist stimulus:* We next tested whether the integrity of lipid rafts is required for the observed molecular dynamics of the AT<sub>1</sub>-R. We used a widely used method, the cholesterol depletion with  $\beta$ -methyl-cyclodextrin (M $\beta$ CD) treatment for lipid raft disruption. In the presence of M $\beta$ CD, the motion of AT<sub>1</sub>-R is greatly altered. The significant BRET signal elevation prior to drop in BRET ratio between KR-YFP and AT<sub>1</sub>-R-luciferase (Fig. 3B, filled rectangles) was totally eliminated in case of M $\beta$ CD pretreatment (Fig. 3B, open rectangles, dotted line). The  $\beta$ -arrestin binding was also affected (Fig. 3C).

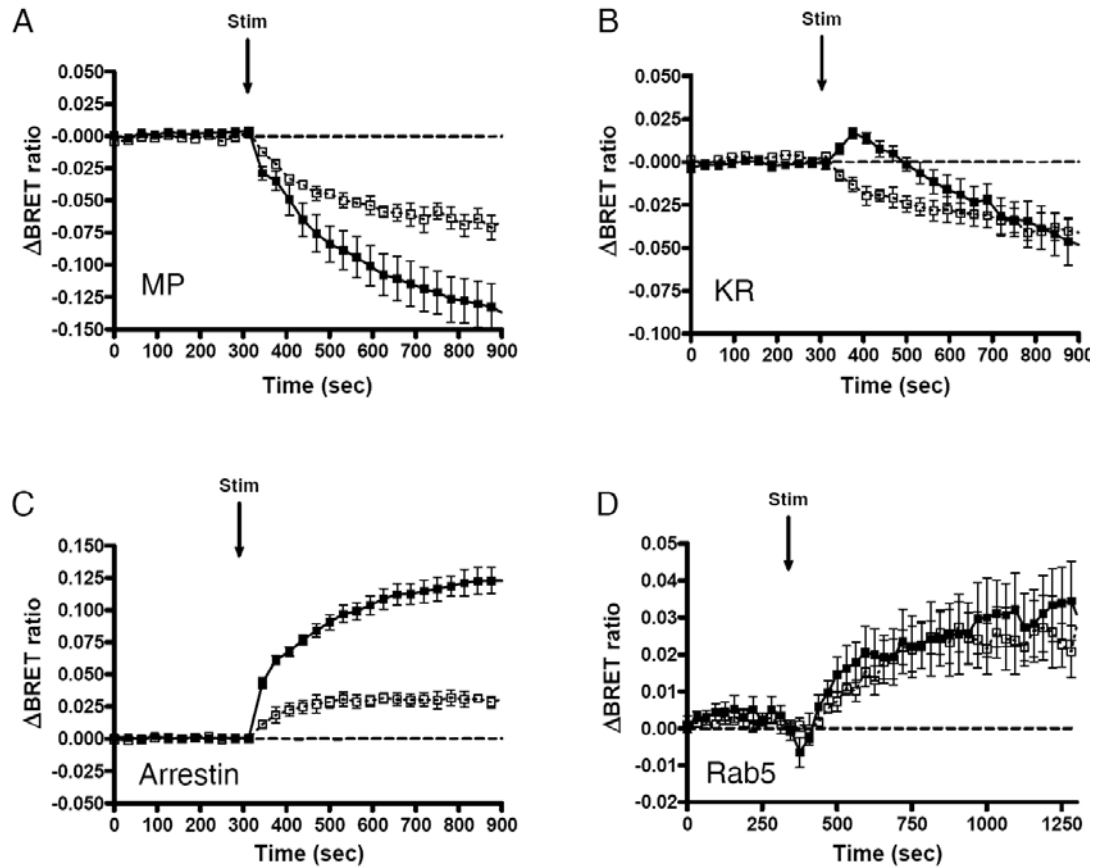


Figure 3. Effect of M $\beta$ CD treatment on the AT<sub>1</sub>-R motion upon agonist stimuli. HEK293 cells were transfected with the plasmids of the AT<sub>1</sub>-R-luciferase and the indicated YFP fused proteins, and after 24 hours the cells were pretreated for 30 min prior to BRET measurement in absence (filled rectangles) or presence of 10  $\mu$ M M $\beta$ CD (open rectangles). After pretreatment the cells were exposed to either 100 nM AngII or vehicle (dashed line) at the indicated time points. The BRET records are average of 3 independent experiments. Mean values  $\pm$  SEM are shown (n = 3).

*Molecular dynamics of AT<sub>1</sub> receptors using various ligands:* We further investigated whether the binding of different ligands to the AT<sub>1</sub>-R causes dissimilar dynamics upon stimulus. The [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII (SII-AngII) is a biased AT<sub>1</sub>-R agonist, which is not able to activate G proteins, but can stimulate G protein-independent mechanisms such as  $\beta$ -arrestin-binding and ERK activation. The stimulus of AT<sub>1</sub>-R with that analogue caused altered dynamics of AT<sub>1</sub>-R compared to AngII (Fig. 4). It is shown in the Fig. 4A and 4B that motion of the AT<sub>1</sub>-R is impaired using SII-AngII, but the receptor is able to be internalized, which is consistent with previous report that the SII-AngII can induce AT<sub>1</sub>-R internalization. It is noticeable that the BRET ratio drop with the MP-YFP is more immediate using SII-AngII stimulus (Fig.

4A, green trace) than using AngII (Fig. 4A, red trace). It is also very apparent that the BRET ratio between KR-YFP and AT<sub>1</sub>-R-luciferase upon SII-AngII treatment decreased immediately, without the initial elevation compared to AngII stimulus (Fig. 4B). It seems that the rapid motion of AT<sub>1</sub>-R-luciferase in response to AngII between plasma membrane markers is G protein-dependent and/or reflects the different conformational change of the receptor using distinct ligands. The AT<sub>1</sub>-R-luciferase binds  $\beta$ -arrestin in less degree by SII-AngII than AngII stimulus (Fig. 4C). The BRET ratio between Rab5-YFP and AT<sub>1</sub>-R-luciferase upon SII-AngII treatment is elevated more significantly by SII-AngII than AngII stimulus (Fig. 4D), which is similar to the results using Rab5-YFP and DRY/AAY AT<sub>1</sub>-R-luciferase upon Ang II (Fig. 1B). Since the SII-AngII stimulus of AT<sub>1</sub>-R does not activate G<sub>q</sub> mediated PtdIns(4,5) $P_2$  breakdown, the BRET ratio with the PLC $\delta$ 1-PH-YFP decreases continually without an initial peak in the drop (green trace, Fig. 4E). The [Sar<sup>1</sup>,Ile<sup>8</sup>]-AngII (SI-AngII) is an octapeptide angiotensin analogue, which is an AT<sub>1</sub>-R antagonist, and was shown earlier that is able to initiate receptor internalization. The stimulus of AT<sub>1</sub>-R-luciferase with SI-AngII also caused altered dynamics of the receptor compared to AngII stimulus (Fig. 4). The results after SI-AngII stimulus of the AT<sub>1</sub>-R-luciferase using either MP-YFP or KR-YFP are similar (green trace, Fig. 4A and B) that supports the idea that proper dynamics of AT<sub>1</sub>-R upon stimulus between plasma membrane markers is G protein-dependent and/or requires physiological conformational change of the receptor. Since the SI-AngII is an AT<sub>1</sub>-R blocker, the stimulus with this analogue does not result PtdIns(4,5) $P_2$  hydrolysis (blue trace, Fig. 4B).

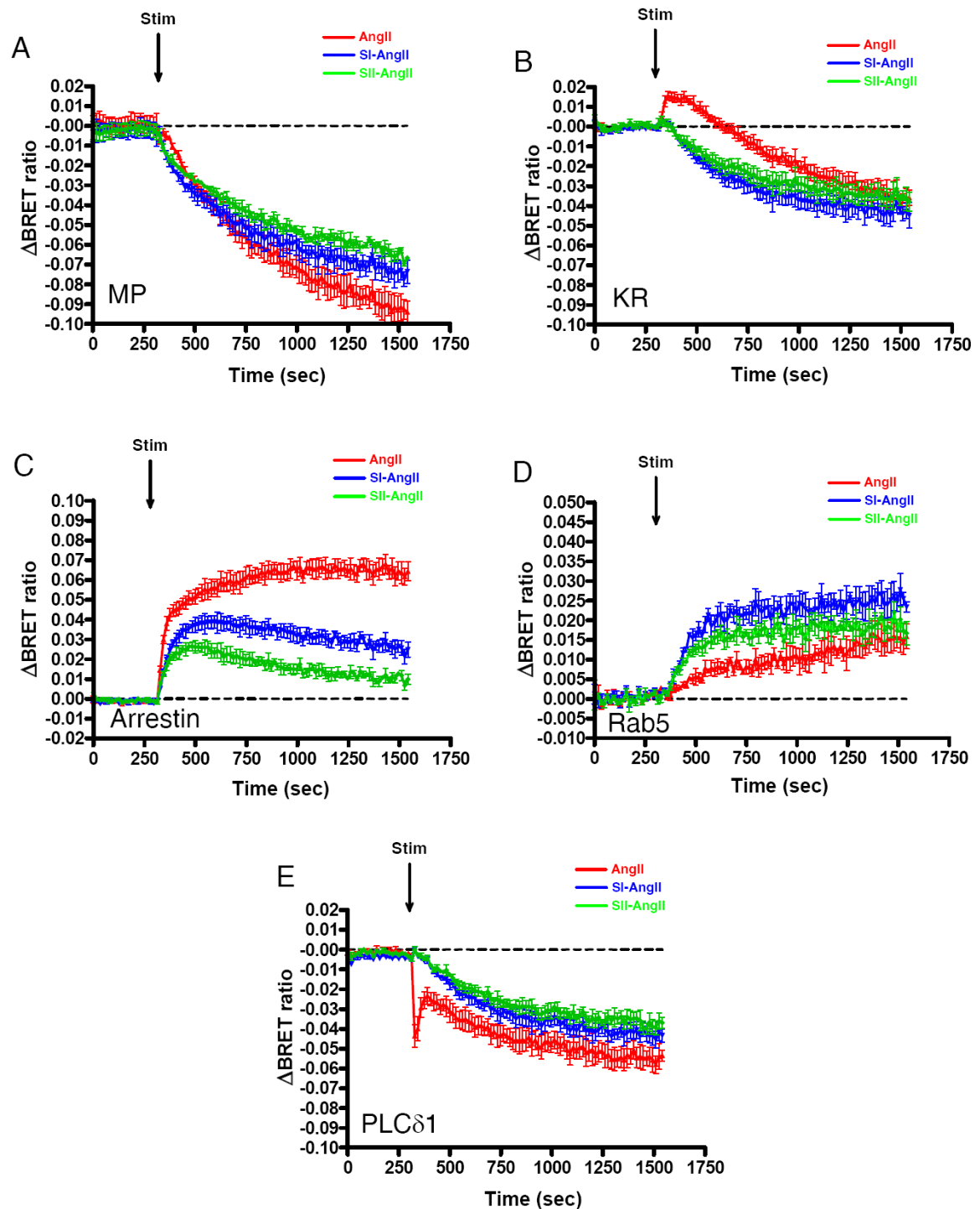


Figure 4. BRET assay between AT<sub>1</sub>-R and different proteins upon stimulus in HEK293 cells. HEK293 cells were transfected with the plasmids of the AT<sub>1</sub>-R-luciferase and the indicated YFP fused proteins, and after 24 hours the cells were exposed to either 100 nM AngII (red trace), or 100 nM SI-AngII (blue trace), or 10  $\mu$ M SII-AngII (black trace), or vehicle (dashed line) at the indicated time points. The BRET records are average of 3 independent experiments. Mean values  $\pm$  SEM are shown ( $n = 3$ ).

*BRET assay for detection of dynamics of 5HT-2C-R and EGF-R:* The preceding results suggest that the molecular dynamics of a plasma membrane receptor could be mapped by the very sensitive BRET technique. Next, we explored our BRET measurements to other receptors in order to test whether we could detect similar dynamics upon agonist stimulus. We measured the motion of 5HT-2C-receptor (Fig. 5) and EGF receptor (Fig. 6) upon agonist stimuli. The 5HT-2CR is also couples to  $G_q$  protein, similarly to the  $AT_1$ -R. After 5-hydroxytryptamine (5HT) binding, the receptor binds to  $\beta$ -arrestin (Fig. 5C), and gets internalized on the basis of BRET ratio elevation between Rab5 and 5HT-2CR (Fig. 5D). The internalization is also apparent using the plasma membrane located MP-YFP and KR-YFP (Fig. 5A and 5B), but we could not detect strikingly different distribution change between using MP-YFP and KR-YFP dissimilar to  $AT_1$ -R. The EGF receptor belongs to the Receptor Tyrosine Kinase class of receptors, and is widely used for the study of receptor internalization. In contrast to  $AT_1$ -R and, which are GPCRs, the EGF-R does not interact with  $\beta$ -arrestin (Fig. 6C), but internalizes upon EFG treatment (Fig. 6D). Similarly to 5HT-2C-R, we also did not observe different distribution change between using MP-YFP and KR-YFP with EGFR-luciferase (Fig. 6A and 6B). Disruption of lipid rafts by M $\beta$ CD pretreatment did not yield dramatic change in the dynamics of either 5HT-2C-R (Fig. 5, open circles) or EGF-R (Fig. 6, open circles).



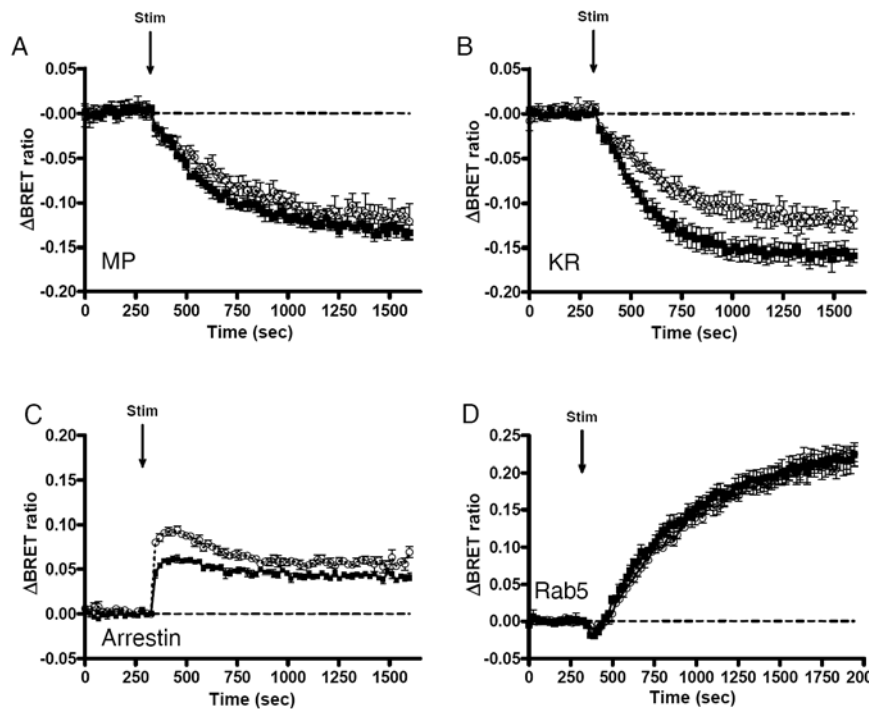


Figure 5. BRET assay between 5HT-2C-receptor and different proteins upon stimulus in HEK293 cells. HEK293 cells were transfected with the plasmids of the 5HT-2C-R-luciferase and the indicated YFP fused proteins, and after 24 hours the cells were pretreated for 30 min prior to BRET measurement in absence (filled rectangles) or presence of 10  $\mu$ M M $\beta$ CD (open rectangles). After pretreatment the cells were exposed to 10  $\mu$ M 5HT or vehicle (dashed line) at the indicated time points. The BRET records are average of 3 independent experiments. Mean values  $\pm$  SEM are shown (n = 3).

Our BRET experiments indicate that the localization of the AT<sub>1</sub>-R rapidly changes after AngII stimulus in HEK293 cells. Comparing the data, which were obtained utilizing either wild type or mutated AT<sub>1</sub>-R co-expressed with the fluorescent probes in HEK293 cells revealed change in distribution between membrane microdomains in response to AngII stimulus. The dynamics of DRY/AYY AT<sub>1</sub>-R, which is not able to activate Gq protein, is dramatically changed compared to wild type AT<sub>1</sub>-R. The stimulus of the DRY/AYY AT<sub>1</sub>-R did not result PtdIns(4,5)P<sub>2</sub> breakdown thus release of PLC $\delta$ 1-PH-YFP from the plasma membrane but the DRY/AYY AT<sub>1</sub>-R internalizes upon stimulus. The  $\beta$ -arrestin binding of the Ang II-bound DRY/AYY mutant receptor is G protein-independent. It seems that the rapid motion of AT<sub>1</sub>-R-luciferase in response to AngII stimulus between plasma membrane markers is G protein-dependent. The BRET ratio between DRY/AYY AT<sub>1</sub>-R-

luciferase and the KR-YFP decreased without the initial elevation compared to wild type AT<sub>1</sub> receptor, and the BRET ratio drop with MP-YFP is more immediate using DRY/AAY AT<sub>1</sub>R-luciferase than using wild type AT<sub>1</sub>R-luciferase. TSTS/A AT<sub>1</sub>-R is not able to bind  $\beta$ -arrestin and its internalization is significantly reduced in response to AngII. We also confirmed these properties of this mutant. Since the internalization of the TSTS/A AT<sub>1</sub>-R is reduced compared to wild type AT<sub>1</sub>-R, the slow decrease component, which is considered as the internalization component, is reduced. We also revealed that the dynamics of AT<sub>1</sub>-R is different using diverse ligands of the receptor. The stimulus of AT<sub>1</sub>-R with either [Sar<sup>1</sup>,Ile<sup>8</sup>]-AngII or [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII caused altered dynamics of AT<sub>1</sub>-R compared to AngII. The biased agonist SII-AngII is not able to activate G proteins, but can stimulate G protein-independent mechanisms such as  $\beta$ -arrestin-binding and ERK activation.

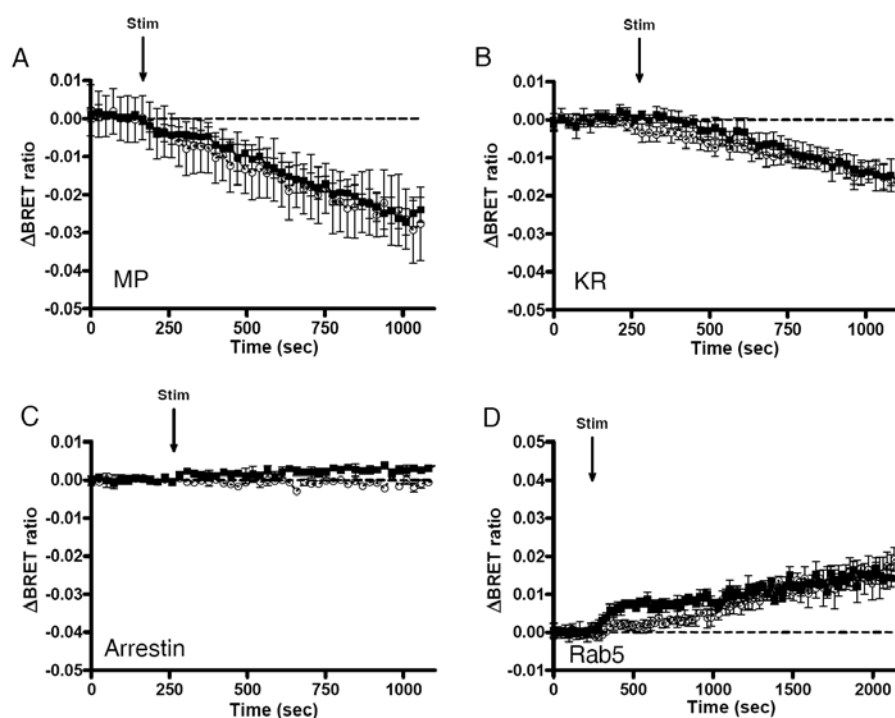


Figure 6. BRET assay between EGF-receptor and different proteins upon stimulus in HEK293 cells. HEK293 cells were transfected with the plasmids of the EGF-R-luciferase and the indicated YFP fused proteins, and after 24 hours the cells were pretreated for 30 min prior to BRET measurement in absence (filled rectangles) or presence of 10  $\mu$ M M $\beta$ CD (open rectangles). After pretreatment the cells were exposed to 50 ng/ml EGF or vehicle (dashed line) at the indicated time points. The BRET records are average of 3 independent experiments. Mean values  $\pm$  SEM are shown (n = 3).

According to our data, the rapid motion of AT<sub>1</sub>-R-luciferase upon stimulus between plasma membrane markers is G protein-dependent or reflects the different conformational change of the receptor using distinct ligands. The binding of an antagonist (SI-AngII) to AT<sub>1</sub>-R caused altered dynamics of the receptor compared to AngII stimulus that supports the idea that proper dynamics of AT<sub>1</sub>-R upon stimulus between plasma membrane markers requires physiological conformational change of the receptor. We have also demonstrated that the dynamics of AT<sub>1</sub>-R upon agonist stimulus is cholesterol-sensitive since it is considerably affected by M $\beta$ CD treatment. These results suggest that the integrity of lipid rafts is required for normal AT<sub>1</sub>-R dynamics. We also examined the molecular dynamics of other receptors, such as 5HT-2C- and EGF receptor. It is important to note that the dynamics of the AT<sub>1</sub>-R is strikingly different from 5HT-2C-R and EGF-R, which shows the usefulness of the BRET based approach to investigate receptor dynamics between membrane microdomains in living cell experiments.

Taken together, these studies provided better understanding of G protein-independent signaling of AT<sub>1</sub>-receptor, and the better understanding of the signaling properties of AT<sub>1</sub>-receptor may provide additional clues to improve the therapeutic potential of drugs that target this receptor which can lead to the development of new pharmacological tools for the treatment of cardiovascular disease. The studies demonstrate the wide scale effects of AngII on Ras activation, and underline that Ras can signal from distinct compartments of cellular membranes to ensure functional diversity but not redundancy of its signaling. We also provided valuable information about the distribution and dynamics of AT<sub>1</sub>-receptor upon ligand binding among membrane microdomains, and revealed G protein-independent mechanisms in the dynamics of the AT<sub>1</sub>-receptor in living cell experiments.